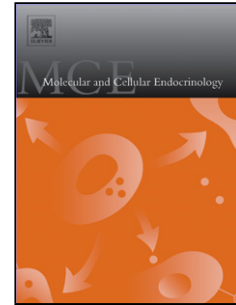


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**GLUCOCORTICOIDS REGULATE THE HUMAN OCCLUDIN GENE THROUGH A SINGLE  
IMPERFECT PALINDROMIC GLUCOCORTICOID RESPONSE ELEMENT**

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**Abstract**

The 65 kDa protein occludin is an essential element of the blood-brain barrier. This integral membrane protein represents an important part of the tight junctions, which seal and protect the blood brain barrier against paracellular diffusion of solutes to the brain parenchyme and are therefore responsible for the high resistance and low permeability between cerebral capillary endothelial cells. However, the molecular basis for the regulation of occludin gene expression is only incompletely understood. In former projects we showed that treatment of a brain microvascular cell line, cEND, with glucocorticoids resulted in increased occludin expression in cell-cell-contacts [Förster, C. et al., J Physiol. 2005 Jun 1;565(Pt 2):475-86.]. Induction of occludin expression by glucocorticoids was shown to be dependent on the glucocorticoid receptor. This study aims to identify the underlying molecular mechanism of gene expression and to identify potential glucocorticoid receptor binding sites within the occludin promoter, the glucocorticoid response elements. We identified one candidate glucocorticoid response element within the distal part of the occludin promoter that differs from the consensus glucocorticoid response element by the presence of a 4-basepair instead of a 3-basepair spacer between two highly degenerate halfsites (5'-ACATGTGTTTACAAAT-3'). Chromatin immunoprecipitation assay and site-directed mutagenesis confirmed binding of the glucocorticoid receptor to this site. The need for glucocorticoid receptor dimerization to induce gene expression was further confirmed by transfection studies using wild type and glucocorticoid receptor dimerization-deficient expression vectors, indicating that transactivation of occludin occurs through the glucocorticoid response element (GRE).

## Introduction

Tight junctions seal the endothelial cell layer and are particularly well developed in endothelia of the blood-brain barrier. Within the central nervous system, tight junctions are highly elaborate and facilitate exchange of solutes and macromolecules and allow leukocyte trafficking [1]. Two different classes of integral membrane proteins constitute the tight junction strands, occludin and members of the claudin protein family [2]. The regulatory mechanisms modulating tight junction gene expression are however only incompletely understood. Several lines of evidence suggest in this context that the tight junction transmembrane protein occludin plays a crucial role in the control of epithelial and endothelial permeability, since tissue expression and content of occludin correlate well with barrier properties [3], and overexpression of occludin increases transendothelial electrical resistance in Madin-Darby canine kidney cells [4]. The difference in occludin levels between brain capillaries and microvessels of non-neural origin [5] suggests that occludin expression is chiefly responsible for the low permeability brain microvascular endothelial cells. It was thus suggested that occludin could be used as a sensitive and consistent marker of functioning tight junctions at the blood-brain barrier.

Epithelial and endothelial barrier function is compromised in many diseases [6], and with few exceptions, the story is one of decreased occludin expression as barrier function is diminished under inflammatory conditions in a wide variety of tissues [7]. For instance, in the colon pathological collagenous colitis presents with decreased transepithelial resistance and decreased expression of occludin and claudin-4 [8]. Comparably, the integrity of tight junctions of the blood-brain barrier is compromised in many disorders of the human central nervous system [9,10]. Neuroinflammation is one of the most severe afflictions, the net effect being increased blood-brain barrier leakiness. Blood-brain barrier leakage has been demonstrated in multiple sclerosis patients, along with discontinuities in immunofluorescent staining patterns for occludin

along brain endothelial cell borders in animal models of this disease [11]. Moreover, systemic peripheral inflammation has been shown to cause leakiness across blood–brain-barrier tight junctions with significant occludin downregulation [12,13], indicating a role for emotional alteration in chronic inflammatory conditions of the CNS [14]. Disruption of occludin staining patterns of blood–brain barrier endothelia is further a hallmark of HIV-associated encephalitis [15]. Finally, aging itself has been linked to declining occludin content in blood–brain barrier endothelia [16].

Recapitulating these diseases correlated with a diminished or altered expression of occludin, the development of approaches to prevent or reverse the downregulation of occludin in CNS pathology may provide a useful therapeutic strategy in the future.

Established therapeutic strategies for several of these diseases include treatment with glucocorticoids [17,18] but the molecular basis of the regulation of blood-brain barrier permeability by glucocorticoids is incompletely understood. Effects of glucocorticoids like hydrocortisone and dexamethasone are known to be mediated by the glucocorticoid receptor [19,20]. The glucocorticoid receptor can bind to specific DNA sequences (glucocorticoid-response elements) in the 5'-flanking region of target genes and transactivate gene transcription [19].

We previously demonstrated barrier tightening effects of glucocorticoid treatment in the cerebral endothelial cell line cEND [5,21]: in an attempt to elucidate the molecular mechanisms of glucocorticoid-induced tightening of the barrier, we were able to show that glucocorticoid signals can directly act at the transcriptional level by interaction with specific cis-acting DNA sequence elements in the occludin gene promoter [21]. More recently, we demonstrated that glucocorticoid-mediated induction of occludin expression [5,13] is confined to endothelial cells

of neural origin (blood brain barrier) while it was not observed in microvessels from non-neural origin. In the present study, we show that the occludin promoter is transactivated by the glucocorticoid receptor in a dimerization- and DNA-binding dependent manner. To define the mechanism of gene regulation, we evaluated the transactivation of a described 5'-gene flanking region (1853 nucleotides) [22] within the human occludin promoter analysing the glucocorticoid-mediated transactivation of sequential promoter fragments and mutant constructs. A putative glucocorticoid response element was identified and characterised to be functional using chromatin immunoprecipitation and site-directed mutagenesis.

## **Material and [23]**

### **Cell cultures**

COS-7 cells were cultured in DMEM medium, HEK293 cells were cultured in MEM medium, both media supplemented with 10% FCS. ~~MCF-7 cells were cultivated in RPMI 1640 medium also supplemented with 10% FCS.~~ All cultures were supplemented with 100 IU ml<sup>-1</sup> penicillin and 100 mg ml<sup>-1</sup> streptomycin (1% PEST). Cells were maintained in an atmosphere containing 5.0 % CO<sub>2</sub> at 37°C.

### **Reporter plasmid construction**

A promoter fragment for the proximal part of the occludin promoter was generated by sequential PCR amplification of pOCLNproLUC\_7 genomic DNA [22] catalysed by *Pfu* polymerase (Stratagene, LaJolla USA). Primers 5'-GTAAACCAAGTT-3' and 5'-GCGCTCTGGACCTG-3' (comp. Table 2) were used in PCR cycling to generate a promoter sequence of 829 bp (-829 to 0). The amplified product was cleaved by *HindIII* and *XhoI*, and the fragment was then cloned into

pGL3-Basic vector (Promega, Mannheim, Germany) using *Escherichia coli* XL\_1Blue cells. The resulting plasmid was then designated pOCLNproLUC\_Prox.

### **Transfection and luciferase assay**

Essentially, transfection and luciferase assays were carried out as described [21]. Briefly, cells were seeded on six-well cell culture plates 24 h before transfection in the specific medium containing 10 % dextran-coated-charcoal (DCC)-treated FCS [24], and 1% PEST at a density of  $2 \times 10^6$  cells per well. Transient transfection experiments utilizing the Effectene reagent (Qiagen, Hilden, Germany) were performed as described by the manufacturer, using 2  $\mu$ g of pOCLN-proLUC7 [22], pOCLN-proLUC\_Prox, pOCLN-proLUC\_Mut, MMTV [23] and 1  $\mu$ g of pTRL-TK (Promega, Mannheim, Germany), and, in the case of COS-7 cells, 0.5  $\mu$ g of wild type human glucocorticoid receptor expression vector pCMVhGR $\alpha$  [25], or 0.5  $\mu$ g of wild type murine glucocorticoid receptor expression vector pCMV5-mGR or 0.5  $\mu$ g of the dimerization-deficient glucocorticoid receptor expression vector A458T [26], respectively, in the absence or presence of ligands (as indicated in the figure legends). To assess glucocorticoid effects on occludin promoter transactivation, after addition of the DNA/Effectene (Qiagen, Hilden, Germany) mixture, cells were incubated overnight at 37°C and 5% CO<sub>2</sub>. After this, 4 ml fresh medium containing 10% DCC-treated FCS/1% PEST and ligands, or vehicle alone were added. After 24 h, cells were washed once with PBS and harvested with 500  $\mu$ l lysis buffer. Thereafter, cellular extracts were prepared and analysed for luciferase activity. Measurement of both, firefly and Renilla luciferase activity was performed with the Dual-Luciferase assay kit (Promega, Mannheim, Germany) according to the manufacturer's instructions. Protein concentration was estimated by standard Bradford protein assay [27]. A 20  $\mu$ l volume of cell lysate was used for assaying the enzymatic activities, using a LB9507 luminometer with dual injector (Berthold, Bad Wildbad, Germany).

Each lysate was measured twice. Promoter activities were expressed as relative light units (RLU), normalized for the protein content and the activity of Renilla luciferase in each extract. The data were calculated as the mean of five identical setups.

### **Semiquantitative PCR**

The PCR reactions were performed in a mastercycler gradient thermocycler (Eppendorf, Hamburg, Germany) under the following conditions: 94°C for 3 min, then 94°C for 30 seconds, followed by 30 seconds of varying annealing (Table 2) temperatures, 72°C for 1 min with depending cycles for each target gene.

PCR products were separated on a 1.5 % agarose gel stained with ethidium bromide. A 100 bp DNA ladder (Generuler, Fermentas, St. Leon-Rot, Germany) was used for calibration. Band densities were compared under UV transillumination.

### **Site directed mutagenesis**

Mutations in the putative distal glucocorticoid response element region were introduced using the Stratagene QuikChange kit (Stratagene, La Jolla, USA), generating the promoter-reporter vector pOCLNproLuc\_Mut.

5'-CATGTGTTTACAAAT-3' was mutated to 5'-CACTTGTTTAAGAAAT-3' using primers as indicated in Table 2. The full-length pOCLNproLUC\_7 was used as a PCR template. PCR was performed amplifying for 18 cycles. Cycling conditions were: 30 sec at 95°C followed by 1 min at 55°C and then 7 min at 68°C. PCR products were digested with the restriction enzyme *DpnI* and transformed into competent *Escherichia coli* XL1\_Blue cells. The mutations were then verified by sequence analysis.

### **Chromatin immunoprecipitation (CHIP) assay**



HEK293 cells ( $0.4 \times 10^6$ ) were seeded on six-well culture plates. After 24 h, cells were transiently transfected with 2  $\mu\text{g}$  pOCLN-proLUC7 [22], pOCLN-proLUC\_Mut according to the manufacturer's manual (Effectene reagent/ Qiagen, Hilden, Germany). 24 hours later cells were treated with 2 ml MEM containing 10% DCC-treated FCS/ 1 % PEST and 1  $\mu\text{M}$  dexamethasone and incubated overnight. The following day cells (approximately  $2 \times 10^6$  cells) were fixed with 1% formaldehyde at 37°C for 10 min. Cells were collected by centrifugation in PBS. The CHIP assay was performed according to the Upstate Biotechnology protocol (Upstate, Charlotteville, USA) with minor modifications. Briefly, samples were diluted with CHIP dilution buffer and precleared with 80  $\mu\text{l}$  of salmon sperm DNA-protein A agarose slurry for 1 h with agitation at 4°C. Immunoprecipitation was performed overnight (12 h) at 4°C with a polyclonal antibody against the glucocorticoid receptor (Santa Cruz Biotechnology, Santa Cruz, USA). After immunoprecipitation, 80  $\mu\text{l}$  of salmon sperm DNA-protein A agarose was added for 1 h at 4°C to capture the immune complexes. Immunoprecipitates were washed five times, with one wash each with low-salt, high-salt, and LiCl buffers and two washes with TE buffer. Immune complexes were eluted twice for 15 min with 1% sodium dodecyl sulfate (SDS) in 0.1 M  $\text{NaHCO}_3$  at room temperature. DNA/protein complexes were heated at 65°C for 4 h to reverse the formaldehyde cross-linking, after which proteinase K was used to digest protein for 1 h at 45°C. DNA was purified by phenol-chloroform extraction and ethanol precipitation and amplified by PCR (primers as listed in Table 2).

**Statistical analysis.** Data are presented as the mean  $\pm$  S.E.M. with  $n = 5$  for each experimental group. Statistical significance between groups was determined by one-way analysis of variance (ANOVA, with Tukey's **post** test). A  $p$ -value  $\leq 0.05$  was considered statistically significant (\*), highly statistically significant at  $p < 0.001$  (\*\*).

## **Results**

### **Occludin expression is dependent on glucocorticoid receptor dimerization**

In the classical pathway transactivation by the glucocorticoid receptor requires binding of the receptor dimers to specific palindromic sequences in the cis-regulatory region of target genes, glucocorticoid response elements [20]. The ability to dimerize depends on the D-loop located in the DNA-binding domain of the glucocorticoid receptor. Several contacts made by D-loop residues at the dimerization interface stabilize receptor dimers and thereby allow cooperative DNA binding [28,29].

Since we could show that treatment with glucocorticoids results in an induction of the human occludin promoter as well as increased occludin expression and protein levels a murine brain capillary endothelial cell line, cEND, as a model of the blood-brain barrier [21] we now examined whether direct DNA binding of the glucocorticoid receptor is necessary for this effect or indirect interactions prevail using a dimerization-deficient glucocorticoid receptor expression plasmid, A458T [26].

Assuming that glucocorticoid receptor homodimerization is required for occludin gene transactivation we compared the transactivation of the human occludin promoter construct in COS-7 cells which do not contain endogenous nuclear receptors, i.e. functional glucocorticoid receptor [30], using a Luciferase-based reporter gene assay after transfection with either the dimerization-competent human wild-type glucocorticoid receptor hGR $\alpha$  [25], the dimerization-competent murine wild-type glucocorticoid receptor [31] or the dimerization-defective

glucocorticoid receptor mutant A458T [26] (Fig. 1B). Using nuclear receptor-free COS-7 cells, detected gene transactivation can be conferred exclusively to the transfected glucocorticoid receptor construct. To control comparability of expression levels of the different GR-constructs we further performed as a control a dose-response curve in COS-7 cells transfected with mGR and hGR $\alpha$  expression vectors at constant ligand concentration (1 $\mu$ M) and a strong and well-described GC-responsive test promoter (MMTV)[23] (Fig. 1A). We tested a concentration range from 5 ng – 5000 ng of the respective GR expression vectors, mGR and hGR, for transfection and subsequent transactivation assay on the MMTV promoter. The dose-response-curve confirms comparability of expression levels for mGR and hGR $\alpha$ : transactivation was shown to be at its optimum for 500 ng hGR and mGR expression vector transfection, and is considerably lower at higher or lower amounts of hGR and mGR expression vector (Fig. 1A). Induction of the occludin promoter was thus assessed after transfection with 500 ng mGR, hGR, and A458T, respectively (Fig. 1B). According to former results treatment of COS7-cells cotransfected with hGR $\alpha$  and the full-length occludin promoter-reporter construct, pOCLN-proLUC\_7 [22], with 1 $\mu$ M dexamethasone stimulated expression of the reporter gene driven by the occludin promoter 2.5-fold ( $\pm$  0.7) compared to the untreated samples (Fig. 1). Treatment of COS7-cells cotransfected with mGR and the full-length occludin promoter-reporter construct with 1 $\mu$ M dexamethasone stimulated expression of the reporter gene driven by the occludin promoter 1.7-fold ( $\pm$  0.3) compared to the untreated samples (Fig. 1)

In contrast there was no transcriptional activation detectable after cotransfection with the dimerization-defective glucocorticoid receptor A458T (Fig. 1), similar to the results found in samples without any receptor transfection (data not shown).

This lacking responsiveness of the occludin promoter in the presence of the dimerization-defective glucocorticoid receptor to dexamethasone treatment indicates that the observed

enhancement of the occludin gene expression is mediated by the dimerized glucocorticoid receptor.

### **Putative glucocorticoid response elements in the occludin promoter**

The glucocorticoid receptor can activate gene transcription by direct binding to simple glucocorticoid response elements. Based on a number of already established glucocorticoid response elements, a consensus sequence has been found as the pentadecameric imperfect palindrome 5'-GGTACAnnnTGTTCT-3' [32]: This canonical response element could not be detected in the 5'-upstream region of the occludin gene. We thus took this approach one step further and tried to deduce a more degenerate glucocorticoid response element sequence which might elicit physiological glucocorticoid response of the occludin gene. For this, we analysed a series of atypical glucocorticoid response elements described in the literature (Table 1): Several studies have identified GR binding sites (glucocorticoid response elements) in a number of target genes [33-35]. These glucocorticoid response elements are not always identical, but show some variability in several nucleotide positions. Nevertheless, Nakabayashi et al. 2001 determined the glucocorticoid response element-consensus sequence to 6 nucleotides in a palindromic repeat separated by three unspecific nucleotides: TCY TGT nnn ACA RGA [33]. We further reconstituted a degenerate full-site glucocorticoid response element from various consensus and imperfect glucocorticoid response elements described in the literature [33-40] and were able to deduce the sequence HHNKGHnnnHCMNNW (H=A/C/T; W=A/T; K=T/G; M=A/C) as a putative degenerate consensus motif. We thus screened a 1853-basepair DNA fragment upstream from the transcription start point of the human occludin gene (Mankertz, 2000) for homology with this degenerate putative glucocorticoid response sequence (Fig.2). Two candidate sequences

with the highest homology to the consensus sequence could be detected (halfsites underlined), one at position -1492 to -1507 5'-ACATGTGTTTACAAAT-3' (hereafter referred as distal putative element) and a second one positioned on -537 to -552 5'-CAATGTTTACACACGA-3' (hereafter designated proximal putative element).

### Localisation of the glucocorticoid response element

For determination of the dose response of the glucocorticoid upregulation of occludin mRNA in cEND BCECs, confluent cEND cells were treated with two different glucocorticoids, the natural ligand hydrocortisone and the synthetic glucocorticoid dexamethasone which has been shown to bind the GR with a 3-fold higher affinity than hydrocortisone and to better stabilize the active GR receptor conformation [41], both at concentrations between  $10^{-6}$  and  $10^{-12}$  M (Fig. 3A). The maximal effect of both, hydrocortisone and dexamethasone, on the induction of occludin mRNA was observed at  $10^{-7}$  M (Fig. 3A) with higher concentrations of glucocorticoids having less effect on the induction of occludin mRNA: the induction of occludin mRNA in cEND cells was shown reach its peak value (about 3-fold) at  $10^{-7}$  M hydrocortisone, and  $10^{-7}$  M of the synthetic specific GR ligand dexamethasone, respectively (Fig. 3A).

The first attempt to examine the putative glucocorticoid response elements was then to compare the occludin induction of the full-length promoter (pOCLNproLUC\_7) (1853 bp, -1853 - 0) and the proximal element (-859 - 0): to test differential transactivation of the two putative glucocorticoid response elements, the proximal part of the occludin promoter, encompassing the putative proximal binding site, was subcloned into pGL3-Basic vector (Promega) (pOCLNproLuc\_Prox). Transactivation of pOCLNproLuc\_Prox was then compared with the full

length occludin promoter pOCLNproLUC\_7 containing both, the putative distal and proximal elements, and, as a negative control, the occludin minimal promoter, pOCLNproLUC\_F [22]. pOCLNproLUC\_F has been described to contain 280 bp of human genomic DNA, from which 208 basepairs are located directly upstream from the transcription start point [22], but no putative glucocorticoid response elements have been located there. As an additional positive control we performed the assay also with the MMTV promoter [23], which contains five canonical glucocorticoid responsive elements.

A luciferase promoter reporter assay revealed the different transactivation levels of the three vectors: after separate transfection of the vectors into HEK293 cells, which in contrast to COS-7 express endogenous glucocorticoid receptor, samples were treated with 1 $\mu$ M dexamethasone and tested for reporter gene transactivation after 24 h (Fig. 3.B).

Corresponding to our former findings the full-length promoter stimulated with 1 $\mu$ M dexamethasone showed a higher enhancement of reporter gene expression compared to the untreated sample (3.4-fold  $\pm$  0.6 induction) (Fig. 3). In contrast pOCLNproLUC\_Prox did not respond to 1 $\mu$ M dexamethasone stimulation (1.0-fold  $\pm$  0.3 induction) (Fig. 3) nor to other dexamethasone concentrations (data not shown), similar to the minimal promoter pOCLNproLUC\_F (0.6-fold  $\pm$  0.1 induction) (Fig. 3.B). MMTV showed the expected strong transactivation (14-fold  $\pm$  2.1) after dexamethasone treatment because of the five glucocorticoid responsive elements, comparing well to the results of other research groups previously described [23].

### **Chromatin immunoprecipitation assay**

To investigate the approximate location of the glucocorticoid response elements more closely we employed the chromatin immunoprecipitation technique. HEK293 cells were transfected with pOCLNproLUC\_7, containing both, the putative distal and proximal elements, and stimulated with dexamethasone. After formaldehyde cross-linking and shearing of chromatin by sonication, receptor/DNA complexes were immunoprecipitated with an anti-glucocorticoid-receptor-antibody. Afterwards PCR was performed with occludin-specific primers spanning the distal putative glucocorticoid response element (-1666 to -1291).

PCR amplification of the fragment spanning the putative element showed that glucocorticoid receptor is recruited to the region where the putative glucocorticoid binding site is estimated after stimulation with dexamethasone, while no immunoprecipitation signals could be observed in samples without glucocorticoid treatment (Fig. 6.A).

### Site directed mutagenesis

To further confirm that the candidate glucocorticoid response element sequence mediates the glucocorticoid responsiveness, the sequence within the wild-type human occludin promoter luciferase construct pOCLNproLUC\_7 was altered by site-directed mutagenesis (pOCLNproLUC\_Mut). The mutation consisted of replacing four basepairs shown to be involved in glucocorticoid receptor binding [32], two in each halfsite, so that also the influence of so-called wobble basepairs could be excluded (Fig. 4).

Both, mutant and wild-type constructs were then transiently transfected into HEK293 cells. Luciferase promoter reporter assay revealed that disruption of the putative glucocorticoid response element sequence abolished glucocorticoid-induced reporter gene expression in response to dexamethasone treatment ( $1.06 \pm 0.09$ -fold transactivation), while the wild-type

construct showed an elevated transactivation level after dexamethasone stimulation ( $1.99 \pm 0.34$ -fold transactivation) following former results (Fig. 5).

The fact that dexamethasone stimulation did not affect gene expression in the mutant vector verified that the distal putative glucocorticoid response element is functional and that hormone binding is not possible after alteration of the sequence. Since glucocorticoid receptor binding to a functional binding site is necessary for transactivation of gene expression we tested whether glucocorticoid receptor binding to the mutated glucocorticoid response element could be detected by CHIP assay: pOCLNproLUC\_7 and pOCLNproLUC\_Mut were separately transfected into HEK 293 cells and treated with  $1\mu\text{M}$  dexamethasone each for the assessment of glucocorticoid receptor binding to the promoter (Fig. 6.B).

pOCLNproLUC\_7 showed a strong CHIP output reflecting glucocorticoid receptor binding to the candidate sequence confirming earlier findings (comp. Fig. 6.A). However, no output could be detected in samples transfected with the mutagenized distal glucocorticoid response element (Fig. 6.B). The glucocorticoid receptor did not bind to the promoter DNA after mutation of the candidate glucocorticoid response element.



## **Discussion**

Since the first therapeutic application in the late 1940s glucocorticoids have been a main focus in endocrinologic studies. Treatment with glucocorticoids is still standard in the therapy of neurological diseases associated with impaired function of the blood-brain barrier. Especially the reduction of brain oedema resulting out of brain tumour [42] or acute ischemic stroke [18] is the main goal achieved by the treatment. Additionally, glucocorticoids play a central role in the handling of multiple sclerosis. However, the management of multiple sclerosis is often disappointing mainly because of the potentially severe side effects and the lacking long-term disease improvement after high-dose glucocorticoid therapy. Probably the better understanding of the molecular mechanisms of glucocorticoid function can result in the development of new therapeutic schemes avoiding some of those disadvantages [11].

In former attempts to elucidate the molecular regulation of glucocorticoid-induced tightening of the blood-brain barrier, we were able to show that glucocorticoids dose-dependently increased transcription of the tight junction component occludin using a murine model of the blood-brain barrier, the cEND brain capillary endothelial cell line [21], as well as a human occludin promoter-reporter construct [22]. These effects were then shown to be associated with increased transendothelial electrical resistance across cEND monolayers and decreased permeability [21], the results in sum pointing to a glucocorticoid receptor-mediated effect on occludin gene expression. In this study, we attempted to elucidate the cellular and molecular basis for glucocorticoid action on occludin gene expression.

Altogether, a variety of different molecular modes contribute to the glucocorticoid control of physiological processes, the regulation of transcription or the modulation of cellular processes by interaction with signalling molecules [11]. For transcription regulation, glucocorticoids exert their effects by binding to the unliganded glucocorticoid receptor localized to the cytoplasm of target cells. Ligand binding induces translocation of the receptor to the nucleus and association with the promoter region of so-called target genes [20,43]. The glucocorticoid receptor then mediates gene expression control by two possible modes, i.e. by direct binding of a receptor homodimer to glucocorticoid response elements in the promoter region of target genes [11,19], or the glucocorticoid receptor may also regulate gene expression by interfering with other transcription factors via protein-protein-interaction without dimerization and direct binding to the DNA [44]. A distinction of the two transactivation modes has become possible by the introduction of a specific point mutation in the dimerization domain of the receptor protein, rendering the formation of a homodimer and thus direct gene transactivation impossible [43]: the glucocorticoid receptor has, like other steroid-hormone nuclear receptors, precise functional and structural domains. Within the DNA-binding domain the P-Box is required for the specific recognition of a response element, while the D-Box is involved in the dimerization of the protein [43]. The DNA-binding domain cannot dimerize itself, but contact with a glucocorticoid response element in the cis-regulatory region of target genes commences this process as an allosteric activator [32]. A single exchange from alanine to threonine in the D-loop domain of the second zinc finger of the glucocorticoid receptor leads to a dimerization-defective receptor which cannot bind to the DNA and is therefore unable to set off direct glucocorticoid receptor-mediated gene induction while indirect protein interactions with other transcription factors still persist [26,45].

In previous work we showed that expression of the tight junction component occludin is regulated by glucocorticoid hormones [5,21]. Aim of this study was to dissect these two possible molecular modes of glucocorticoid-mediated transcriptional control over the occludin gene and to identify the transcription enhancing glucocorticoid response elements. We were able to show that glucocorticoids upregulate occludin gene expression by directly binding to the occludin promoter in a dimerization-dependent manner: by use of wild type and dimerization-defective glucocorticoid receptor expression vectors [45] we were able to demonstrate that this promoter transactivation is dependent on dimerization of the glucocorticoid receptor.

Then, by sequence analysis and chromatin immunoprecipitation of promoter fragments of varying length we delineated a functional highly degenerated glucocorticoid response element in the distal part of the occludin promoter at position -1507 and -1492. Transient transfection of a 1.85-kb human occludin 5'-upstream region fused to the reporter luciferase identified that the element controlling the glucocorticoid-mediated promoter activation was in fact located within this region of the gene. We then confirmed glucocorticoid receptor binding to the DNA in the occludin 5'-upstream gene region using the chromatin immunoprecipitation assay to confirm DNA binding of the glucocorticoid receptor by detection of receptor-DNA-complexes [46]. By subcloning strategies of promoter fragments of varying length we could narrow down this glucocorticoid-responsive region to nucleotides -1853 – 859 in the distal part of the occludin promoter fragment. Promoter-reporter gene assays and chromatin immunoprecipitation analysis of promoter fragments of different length we could demonstrate the putative glucocorticoid response element must reside in the distal part of the promoter between nucleotides -1853 and -859.

For further confirmation site-directed mutagenesis was performed according to other authors investigating corticosteroid receptor binding sites [35,40]. Site-directed mutagenesis of this region confirmed that the binding element resides between -1507 and -1492 from the

transcription start site. These data suggest that the most potent steroid response element within the occludin promoter is located at position -1507 and -1492 from the transcription start site.

This pentadecameric sequence contains an asymmetric imperfect glucocorticoid response element that was not previously identified by searching with transcription factor databases [46]. Based on a number of glucocorticoid response elements the consensus sequence had been defined to be the pentadecameric imperfect palindrome 5'-GGTACAnnnTGTTCT-3' [47] in which the 3' half is most conserved while the 5' shows a high degree of flexibility which does not imply a reduced glucocorticoid response [32]. The 3-basepair spacer between both half-sites was shown to be a strict requirement for cooperative binding to the palindromic glucocorticoid response element [28]. However, several glucocorticoid response elements differ from this consensus sequence but have been shown to be biologically functional notwithstanding the deviation (Table 1), so that the detection of biological effects justifies the search after non-canonical glucocorticoid response elements in potential target genes [48]. In the past, multiple glucocorticoid receptor binding sites in an unusual configuration were found in *cis*-acting sequences mediating the delayed and secondary response to glucocorticoids, so-called secondary glucocorticoid response elements [49,50]. They represent clusters of glucocorticoid response element half sites with irregular spacing, oriented both head to head and head to tail, both in the alpha-globulin gene and the promoters of genes encoding the milk proteins whey acidic protein (WAP) and beta-casein [49,50]. The authors emphasized however the importance of specific cellular factors, which facilitate the binding and/or transactivation of these gene promoters by the steroid receptor. The presence and requirement for such (tissue) specific factors for the induction and transactivation of the occludin gene by glucocorticoids remains to be elucidated in the future.

Interestingly, we detected a second asymmetric putative glucocorticoid response element was detected between nucleotides -537 to -552 within the occludin promoter which did however not bind to the glucocorticoid receptor protein as assessed by chromatin immunoprecipitation, so that its transactivation might require the interaction of glucocorticoid receptor with other transcription factors in the cellular context [11]. The upstream region of the human occludin gene further contains several canonical glucocorticoid responsive half-elements [21], which generally are insufficient by themselves to confer hormone responsiveness, since two palindromic half-sites (AGAACA) separated by a three-base pair spacer have been shown to be required to bind a receptor dimer and activate promoter activity [19].

The mechanism, magnitude, and even the polarity (positive or negative) of transcriptional regulation are determined by the sequence and architecture of the response element and promoter and the availability and activities of regulatory factors that can function at that response element [51]. In this report, we attempted to delineate the molecular background for reported biological glucocorticoid receptor function at the blood-brain barrier [11,18] and were able to identify a highly degenerate putative glucocorticoid response element, which was subsequently shown to bind the liganded receptor. Although the described response element is highly degenerated, a physiological effect has been observed [11]. It is known that the glucocorticoid receptor activates or represses transcription depending on the sequence and architecture of the glucocorticoid response elements in target genes and the availability and activity of interacting cofactors [51]. The element detected is highly degenerated at positions  $\pm 6$ - $\pm 4$ , but partially symmetric at positions  $\pm 1$  -  $\pm 3$ . Available data indicate in this context that under physiologically relevant conditions, glucocorticoid receptors have high selectivity and affinity only for DNA containing specific partially symmetric glucocorticoid response elements and further suggest that this high

affinity for such DNA sites may be sufficient to account for the selective regulation of gene expression observed in glucocorticoid-responsive cells [52]. In particular, the role of different functional surfaces on the receptor itself in regulating its targets is unclear, due in part to the paucity of known target genes. These findings can thus have implications for the future search of more gene response elements mediating glucocorticoid action and signalling, the composition and structure of regulatory complexes and the mechanisms of context-specific transcriptional regulation. Future studies will have to elucidate the composition of the distinct regulatory complexes assembled at the occludin glucocorticoid response element identified in its normal chromosomal setting to define the determinants of tissue-specific glucocorticoid action.

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### **Abbreviations**

FCS (fetal calf serum), CHIP (chromatin immunoprecipitation)

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### Figure legends

**Fig. 1. GR-dependent transactivation of occludin after hGR $\alpha$ , mGR and GRdim transfection and dexamethasone treatment.**

**(A)** To compare expression levels of mGR and hGR $\alpha$  we co-transfected COS-7 cells with MMTV-luc, the Renilla luciferase reporter vector pRL-TK (Promega) for normalization of transfection efficiency and either the expression vector for human glucocorticoid receptor, pCMVhGR $\alpha$  (hGR $\alpha$ ) or the expression vector for murine glucocorticoid receptor (pCMV5-mGR)(mGR). mGR and hGR $\alpha$  expression vectors were transfected in a concentration range from 5 ng – 5000 ng of the respective GR expression vectors and MMTV-luc transactivation tested at constant ligand concentration (1  $\mu$ M). Transactivation was detected to be at its optimum for 500 ng hGR and mGR expression vector transfection, respectively, and is considerably lower at higher or lower amounts for both, hGR and mGR expression vectors.

**(B)** COS-7 cells were cotransfected with pOCLNproLUC\_7, the Renilla luciferase reporter vector pRL-TK (Promega) for normalization of transfection efficiency and 500 ng of either expression vector, human glucocorticoid receptor, pCMVhGR $\alpha$  (hGR), the expression vector for murine glucocorticoid receptor (pCMV5-mGR) or the dimerization deficient glucocorticoid receptor A458T (GRdim). Cells were then maintained in serum reduced (2 % FCS) medium with or without 1 $\mu$ M dexamethasone (dex) for 24 h. Afterwards, cell extracts were tested for luciferase and Renilla activity.

**Fig. 2. Putative glucocorticoid receptor binding sites in the human occludin promoter.** The human occludin promoter contains two degenerate putative full-site glucocorticoid responsive element located at position -1492 to -1507 5'-ACATGTGTTTACAAAT-3' (distal putative

element, distal GRE) and -537 to -552 5'-CAATGTTTACACACGA-3' (proximal putative element, proximal GRE) on which glucocorticoids could influence gene expression.

**Fig 3. A. Induction of occludin mRNA transcription by glucocorticoids in cEND cells.**

cEND cells were incubated with the glucocorticoid hormones hydrocortisol (HC) and dexamethasone (Dex) for 3 days. Dependence of occludin mRNA induction on the concentration of glucocorticoids was determined in the presence of  $10^{-6}$  –  $10^{-12}$   $\mu$ M HC or Dex, respectively. A maximum of glucocorticoid effects on occludin mRNA transcription was observed at  $10^{-7}$  M HC and Dex, respectively.

**Fig. 3. B. Transactivation of the occludin promoter after treatment with dexamethasone compared to the unstimulated vector.** HEK 293 were cotransfected with pRL-TK and either pOCLNproLUC\_Prox (proLuc\_prox), pOCLNproLUC\_F (proLuc\_F) or pOCLNproLUC\_7 (proLuc\_7) and MMTV as a positive control. After 24 h, cells were stimulated with or without dexamethasone, harvested after 24 h and the promoter activity was assessed by luciferase promoter reporter assay.

**Fig. 4. Site directed mutagenesis**

Mutations were generated using the Stratagene QuikChange kit. PCR primers were designed to convert TG in the first hexamer into CT and CA in the second into AG. The 1853 bp human occludin promoter fragment pOCLNproLUC\_7 was used as template.

**Fig. 5. Comparison of the wild-type occludin promoter and the mutated occludin promoter.**

Reporter gene expression was analyzed after transient transfection of pOCLNproLUC\_7 (proLuc\_7) and pOCLNproLUC\_Mut (proLuc\_Mut) into HEK 293 cells and dexamethasone (dex) treatment. After 24 h, luciferase promoter reporter assay was performed.

**Fig. 6A** Dexamethasone induces glucocorticoid receptor association with the distal part of the occludin promoter. HEK293 were transfected with pOCLNproLUC\_7 and were untreated (lane 1 and 3) or treated (lane 2 and 4) with dexamethasone (dex) for 24 hours. Cells were harvested and the chromatin was sonicated. The fragmented DNA was immunoprecipitated with the anti-glucocorticoid receptor antibody (GRab) (output, lane 3 and 4). Some fragmented chromatin was saved as an input before precipitation (input, lane 1 and 2). DNA from input and output was amplified by PCR with primers including the distal putative glucocorticoid response element . The resulting products were analyzed on a 1.5% agarose gel.

**Fig. 6B CHIP assay with mutated occludin promoter construct.** HEK293 were transfected with pOCLNproLUC\_mut and were untreated (lane 1 and 3) or treated (lane 2 and 4) with dexamethasone (dex) for 24 hours. The fragmented DNA was then immunoprecipitated with the glucocorticoid receptor antibody (GRab) (output, lane 3 and 4). Some fragmented chromatin was saved as an input before precipitation (input, lane 1 and 2). DNA from input and output was amplified by PCR with primers including the distal putative glucocorticoid response element . The resulting products were analyzed with a 1.5% agarose gel.

## Figures and tables

Figure 1

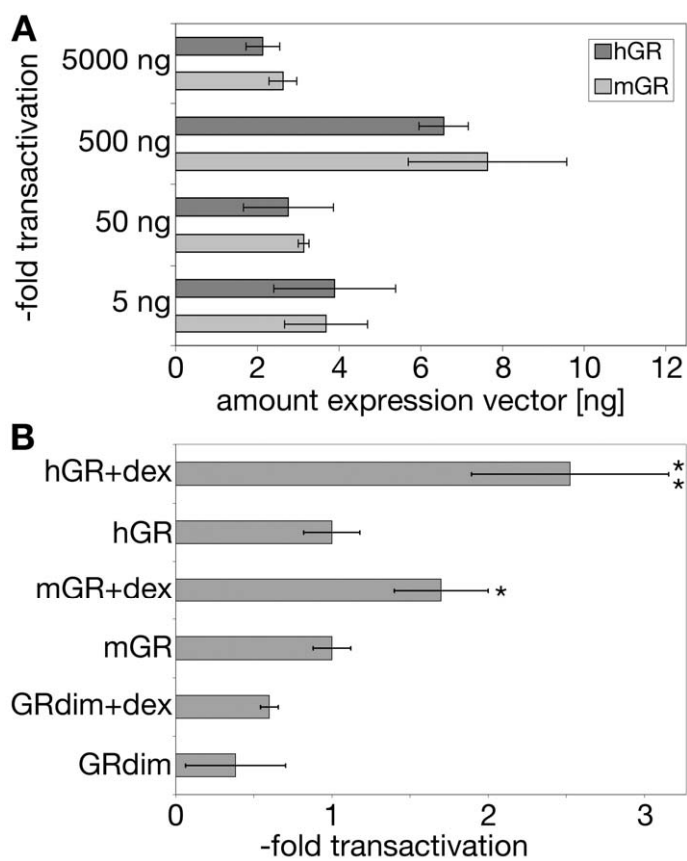


Figure 2

ATTATGCTAAGTGAAAGAAGACAGACACAAAGGCACATATTGTATTATCCATTCATATAAAATGTCCAG -1783  
 AATAGGCAATTAATAGAGACAGAAAGTACATTAGTGGTTGCCGAGGGATGAGGGGAGAGGGGAAATGGG -1713  
 GAGTGACTATTAATGGGCATGGGGTTCTTTTGGGGCCGATTAATAATGGAATTAGATGGTGGTGATGGTT -1643  
 GTACAATCTGGTGAGTATACTAAAACCCATTGAATTGTACGTGCCCTTTAAAAGGGTGAATTTTATGGTA -1573  
 TGTGAACCTCTCTTTATAAAGTGAAAAAACTCCTAAGATCTCTTCAGAACATGTCAAAATACATTATA -1503  
 AAATAATAGACATGTGTTTACAAATCTGAGAGTATTAGGAAATGTTCCCTTGTTAATTATAAACCAAAT -1433  
 distal GRE  
 GGAATGTTGGAGATTTTCAGTAATCTGACAGGGAACATTAAGGGGATTAACCTGACTTCCCAGTGTTAA -1363  
 CAATACCAATTTAACTGCATCATTCAAAACTACATAGTCATATTAAGCATTTGTAGCAATGACTTCC -1293  
 ACGAAAAAATACCAATTAATTAATTAACCCATTAAGCTGCCATCATCTGAAATACCTCATATTTATAT -1223  
 AGTGCTTTTACTTCCTCAAGTATGAATTGTGCCTTAAGATCTAATGTATGGGAGAGTCACATCTTAACC -1153  
 ATTTAATTAAGGTAGAGAAGTGGGTGGGATGGATAGAAATTTATAGCAATGCTGACATTCAGATTG -1083  
 GAACACAAAGACAAGCAGGATGTAAAGAACCTAAAAGTTCGCTTTCATGCAGATAGTTAAATGCCAAGA -1013  
 ACTATAAATGCCACATCCTGGAGTACAATTAATAAATATGTTGAAAAACAACCAACATACATAAAAAATAT -943  
 ACACAGTGTAAAGTGCAGACTATGAAATTTCCCTTGGAACAGAATCCAGATCAAGAAATAGAATACAGC -873  
 ACCCGGAACAAAGTGCTTTTTCGTTTCTTAAAAAAGTAAACCAAGTTTATTTGCTTTTAA -803  
 GTAGTGTGTTCTTAAAGCACTACAGGTGGTAAACAACGTATAAGGTGCTTTTCTCAGGCCAAAGAGC -733  
 CATAATAGTTTAAATTTCCCGGTCTCAAGAGCGGACCGGGTGGCAGGAGAGGCCCTGGGGTGGTGAT -663  
 GTGTAACTGTATTATGCACCTTAGCCTGCTGGATGGCAACTAACACCTACAGTAGTTCACCTCATTTT -593  
 AACCCCTCTAAGTAATTGTCTCTTATTCTGAATCTAGAGATTCAGAAACAGCGC CAATGTTTACACAGGA -523  
 proximal GRE  
 CTTTGGAAATTTTCCAGGAGTCTTTTCGTTGGAGCAATACATCTAGATGCCTTTTCCAGCAACAGTTTA -453  
 ATCAAATCTGGAAGCAGAAAAGTCTCCTGTGAGGACGTGCCTTTCCTATCAAAGTGTGAGTGCCTGGA -383  
 CCCTCTTCCGGAGGAAACAGTCCCTCTGGACCTCGTTCGGCCTCTCTCCATCAGACACCCCAAGGTTT -313  
 CATCGAAGCAGGCGGAGCACCGAACGCACCCCGGGTGGTCAGGGACCCCATCCGTGCTGTCCCTAG -243  
 GAGCCCGCGCTCTCCTCTGCGCCCGCTCTCGGGCCGCAACATCGCGGGTTCCTTTAACAGTGCCT -173  
 GGCAGGTGTGGGAAGCAGGACCGCTCCTCCGCCCTCCATCCGAGTTTCAGGTGAATTGGTCACC -103  
 GAGGGAGGAGCCGACACACCACCTACACTCCCGCTCCACCTCTCCCTCCCTGCTTCCCTGGCGGA -33  
 GGCGGCAGGAACCGAGAGCCAGGTCCAGAGCGC 0

Figure 3

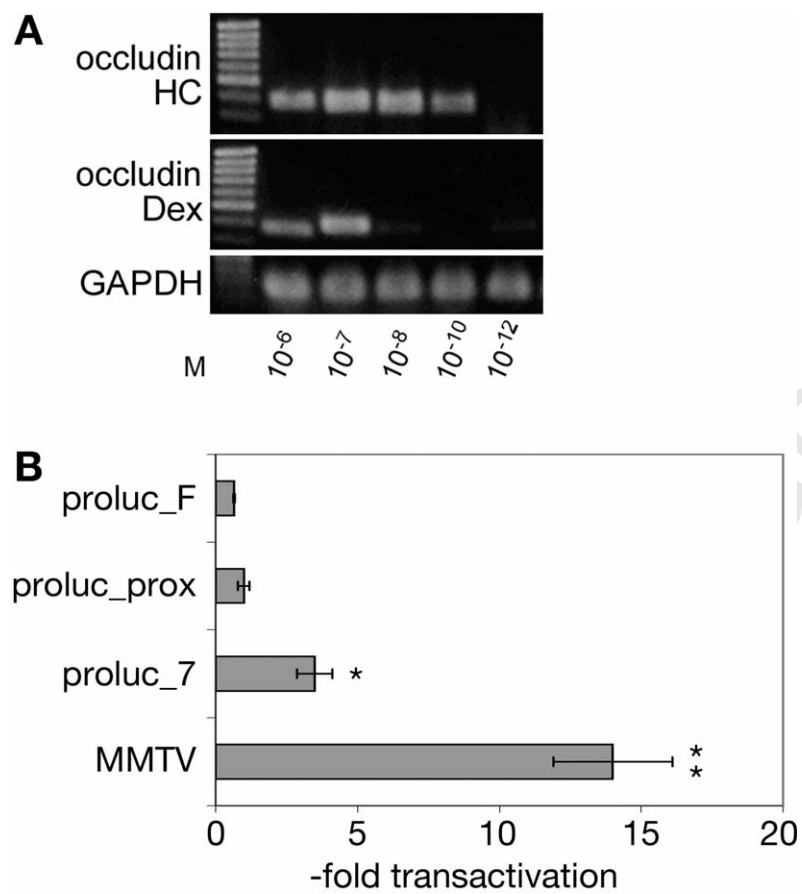




Figure 4



Figure 5

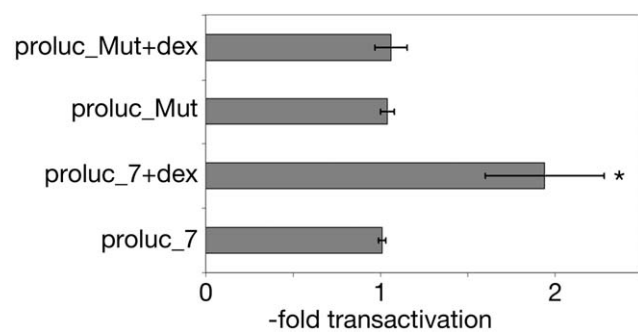


Figure 6

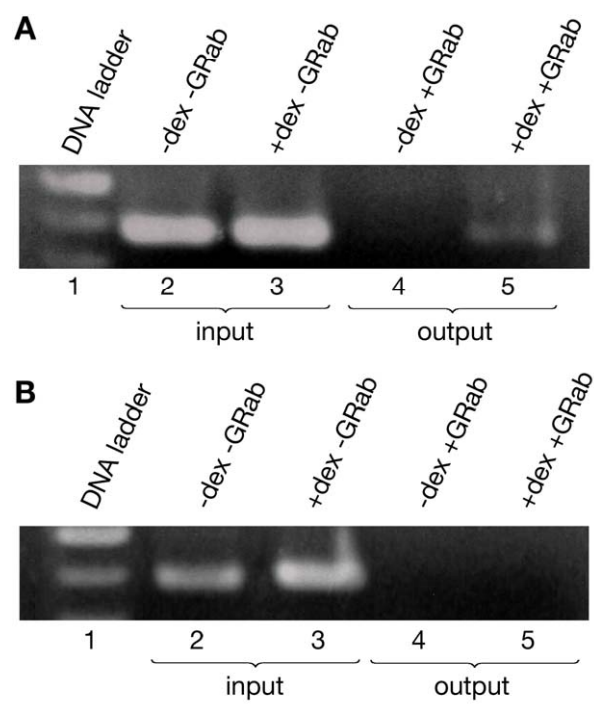


Table 1

glucocorticoid-responsive element $\alpha$	gene $\alpha$	ref. ....
NCN <b>TGT</b> · ... · <b>ACANG</b> $\alpha$	somatostatin-receptor-type-2 $\alpha$	(31).....
TCCT <b>TGT</b> · ... · <b>ACAACG</b> $\mathbb{I}$	rat-aldehyd-dehydrogenase $\alpha$	(32).....
CAAT <b>TGT</b> · ... · <b>ACAAGA</b> $\alpha$		
TCN <b>TGT</b> · ... · <b>ACANGA</b> $\alpha$	rodent-alpha-fetoprotein $\alpha$	(33).....
CCT <b>TGT</b> · ... · TCCCGT $\mathbb{I}$	androstane-receptor $\alpha$	(34).....
<b>ACGGAA</b> · ... · <b>ACAAGG</b> · $\alpha$		
<b>ACTTGA</b> · ... · <b>ACA</b> AAA $\mathbb{I}$	CYP2C19 $\alpha$	(35).....
TTTT <b>TGT</b> · ... · TCAAGT $\alpha$		
TCGCGT · ... · <b>ACACGT</b> $\mathbb{I}$	rat-Na <sup>+</sup> K <sup>+</sup> -ATPase-beta-1 $\alpha$	(36,37)
<b>ACAGGC</b> · ... · CCAAGT $\mathbb{I}$		
<b>ACATGT</b> · ... · <b>ACAAGA</b> $\alpha$		
<b>ACATGT</b> · ... · <b>ACAACA</b> $\alpha$	CYP2C9 $\alpha$	(38).....

Tab. 1: literature comparison of known glucocorticoid response elements

Bold nucleotides highlight the similarities with the canonical element.

Table 2

primers <sup>α</sup>	target and primer sequences <sup>¶</sup>	product size <sup>α</sup>	annealing temperature <sup>α</sup>
<sup>α</sup>	<u>subcloning<sup>¶</sup></u> pOCLNproLUC_Prox <sup>α</sup>	<sup>α</sup>	<sup>α</sup>
F <sup>¶</sup> R <sup>α</sup>	5' - GTAAACCAAGTT - 3' <sup>¶</sup> 5' - GCGCTCTGGACCTG - 3' <sup>α</sup>	829 <sup>α</sup>	47°C <sup>α</sup>
<sup>α</sup>	<u>ChIP assay distal glucocorticoid responsive element<sup>α</sup></u>	<sup>α</sup>	<sup>α</sup>
F <sup>¶</sup> ¶ R <sup>α</sup>	5' - TGGAATTAGATGGTGGTG - 3' <sup>¶</sup> 5' - TGTAGCAATGACTTCCAC - 3' <sup>α</sup>	375 <sup>α</sup>	53°C <sup>α</sup>
<sup>α</sup>	<u>site directed mutagenesis distal glucocorticoid responsive element<sup>α</sup></u>	<sup>α</sup>	<sup>α</sup>
F <sup>¶</sup> ¶ R <sup>α</sup>	5' - GGGAACATTTCTAATACTCTCAGA <sup>¶</sup> TTCTTAAACAAGTGTCTATTATTTT <sup>¶</sup> ATAATGTATTTTGAC - 3' <sup>¶</sup> 5' - GTCAAATAACATTATAAAATAAT <sup>¶</sup> AGACACTTGTTTAAGAATCTGAGAGT <sup>¶</sup> ATTAGGAAATGTTCCC - 3' <sup>α</sup>	65 <sup>α</sup>	55°C <sup>α</sup>

Tab. 2: Primers used for PCR